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Brain Research 905 (2001) 178–187

BRAIN RESEARCH

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Research report

Involvement of the cerebellar adenosine A_1 receptor in cannabinoid-induced motor incoordination in the acute and tolerant state in mice

K.P. DeSanty, M. Saeed Dar*

Department of Pharmacology, Brody School of Medicine, East Carolina University, Brody Medical Sciences Building, Greenville, NC 27858, USA

Accepted 10 April 2001

Abstract

Cannabinoids are known to impair motor function in humans and laboratory animals. We have demonstrated an accentuation of cannabinoid (CP55,940)-induced motor incoordination in mice by the adenosine A_1 receptor-selective agonist N^6-cyclohexyladenosine (CHA) (4 ng) using an intracerebellar (ICB) microinjection method. This effect was mediated by the A_1 receptor because pre-treatment with ICB 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (100 ng), an adenosine A_1 receptor selective antagonist, completely abolished the accentuation. Furthermore, ICB pre-treatment with DPCPX (100 ng) before ICB CP55,940 (15 μg) attenuated the motor incoordination suggesting a modulation by an endogenous adenosine A_1 system. ICB microinjection of CHA or DPCPX prior to ICB vehicle had no effect on normal motor coordination. ICB microinjection of dipyridamole (25 μg), an adenosine transport inhibitor, significantly accentuated the motor incoordination by ICB CP55,940 (15 μg), providing further support for the involvement of endogenous adenosine in the action of CP55,940. Tolerance to the motor incoordinating effect of ICB CP55,940 was demonstrated following 3 days of i.p. CP55,940 (0.1, 1 or 2 mg/kg every 12 or 24 h; total of six or three injections, respectively). Interestingly, animals which exhibited tolerance to ICB CP55,940 also demonstrated tolerance to the accentuating effect of ICB CHA suggesting cross-tolerance between adenosine agonists and cannabinoids. Cross-tolerance was also demonstrated following 3 days of i.p. CHA (0.25 or 1 mg/kg every 24 h; total of three injections) as further evidence of the modulatory role of the cerebellar adenosine system in the acute manifestation of CP55,940-induced motor incoordination. The involvement of cerebellar adenosine and the A_1 receptor in cannabinoid actions is circumstantially supported by previous evidence that CB_1 receptors and A_1 receptors are both localized on cerebellar granule cell parallel fiber terminals and basket cell neurons where they serve to inhibit the release of neurotransmitters.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Behavioral pharmacology

Keywords: Cannabinoid; Ataxia; CB_1 receptor; Adenosine A_1 receptor; Cerebellum

1. Introduction

Cannabinoids are known to impair motor function in humans and laboratory animals [1]. We have previously demonstrated that Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of marijuana, impairs motor coordination through a cerebellar CB_1 receptor mechanism in mice [7]. This behavioral effect of Δ^9-THC was not surprising due to previous evidence of dense CB_1 receptor localization in the cerebellum and the well-known function of the cerebellum in coordinating movements [19].

Autoradiographical studies have demonstrated a presynaptic localization of CB_1 receptors on cerebellar granule cell axonal terminals in the molecular layer of the cerebellum [19,23]. It is likely that these receptors inhibit glutamate release from the neuronal terminal upon activation by cannabinoid agonists such as CP55,940 and anandamide [19]. There is also evidence for localization of CB_1 receptors on the inhibitory basket cells and excitatory climbing fiber terminals which synapse onto Purkinje cell bodies [23,33]. Several inhibitory presynaptic receptors appear to be co-localized with CB_1 receptors such as the $GABA_B$, adenosine A_1 and κ opioid. As for these re-

*Corresponding author. Tel.: +1-252-816-2885; fax: +1-252-816-3203.

E-mail address: darm@mail.ecu.edu (M.S. Dar).

0006-8993/01/$ – see front matter
PII: S0006-8993(01)02533-1

ceptors, the CB_1 receptors are $G_{i/o}$ protein-coupled to inhibit adenylate cyclase and modulate the conductance of Ca^{2+} and K^+ channels [1,12,17,20,21]. In vitro studies have demonstrated additive GTPase activity when $GABA_B$ and A_1 agonists are co-incubated [35] and also when CB_1 and $GABA_B$ agonists are co-incubated [26]. It was concluded that these receptors may couple to their own pool of G-proteins but converge to inhibit common adenylate cyclase enzymes [26]. Such an interaction is plausible between CB_1 and A_1 receptors.

The purpose of our study was to expand on our previous work which demonstrated a functional interaction between CB_1 and A_1 receptors in the cerebellum of male CD-1 mice using motor incoordination as the behavioral test. We have recently demonstrated accentuation of Δ^9-THC-induced motor incoordination by the adenosine A_1 selective agonist, N^6-cyclohexyladenosine (CHA), through a cerebellar mechanism [7]. The dose of CHA utilized was too low by itself to induce motor incoordination but significantly accentuated the motor incoordination by intracerebellar (ICB) Δ^9-THC when co-administered. Furthermore, ICB administration of the selective A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), significantly attenuated the motor incoordination induced by Δ^9-THC.

The purpose of the present investigation was to establish whether the synthetic cannabinoid agonist, CP55,940, shares the same interaction with the adenosine A_1 receptor system when microinjected directly into the mouse cerebellum. We also set out to extend present and previous research by hypothesizing that tolerance to intracerebellar cannabinoid-induced motor incoordination occurs following repeated systemic administration. As further support of the adenosine A_1 receptor involvement in cannabinoid-induced motor incoordination, we tested for a cross-tolerance effect with the A_1 selective agonist, CHA, by daily systemic dosing for 3 days with CHA followed by an ICB CP55,940 challenge. We suggest that the acute and chronic interactions between drugs acting through CB_1 and A_1 receptors may result from anatomical co-localization and overlap in their signal transduction cascade.

2. Materials and methods

2.1. Animals

Male CD-1 mice were purchased from Charles River Labs (Raleigh, NC). The mice were 5–6 weeks old and weighed between 23 and 28 g at the time of the behavioral experiments. The mice were maintained in a housing facility under controlled humidity and temperature (23–25°C) and kept on a 12:12-h light/dark cycle. Each animal was housed in its own individual plastic cage. Mice had free access to water and commercial mouse chow. All experiments were evaluated and approved by the Animal Use and Care Committee of East Carolina University.

2.2. Stereotaxic surgery

Two days after arrival, mice were anesthetized with chloral hydrate (450 mg/kg, i.p.) prior to placement in a small stereotaxic frame (David Kopf Instruments, CA). The head of each mouse was trimmed of hair, scrubbed with povidone–iodine by swab stick and then wiped clean with an isopropyl alcohol swab. With the skull flat, a 2-cm long mid-sagittal incision was made by sterile scalpel in order to expose the skull. Cannulation of the cerebellum was performed aseptically according to the following coordinates of Slotnick and Leonard [30]: AP −6.4 mm (from bregma); ML ±0.8 mm; DV −1.0 mm from the brain surface. The stainless steel guide cannula (22 gauge, 10 mm length) was lowered through a drilled craniotomy hole into the superficial layers of the anterior lobe region of the cerebellum. Durelon cement (Premier Dental Products Co., Norristown, PA) was used to anchor the cannula to the skull surface. A removable stainless steel wire plug was placed inside the guide cannula to prevent occlusion. Following surgery, each animal received 3000 units, subcutaneously, of Durapen (procaine and benzathine penicillin G, Vedco, MO) to prevent infection during post-surgical recovery. Each animal also received an injection of ketorolac tromethamine (Abbott Labs, N. Chicago, IL), 2 mg/kg i.p., for analgesia shortly after surgery and again 4–6 h later. Animals were allowed to recover from surgical trauma and anesthetic effects in their own individual cages at the Brody School of Medicine animal care facility for a minimum of 5 days before behavioral testing.

2.3. Drugs

Drug solutions were prepared the day of use in behavioral experiments. The following drugs were used in experiments: cannabinoid receptor agonist CP55,940 (a gift from Pfizer, Groton, CT); adenosine agonist, N^6-cyclohexyladenosine (CHA); adenosine transport inhibitor, dipyridamole and A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (all obtained from RBI, Natick, MA). CP55,940 was dissolved in 100% dimethylsulfoxide (DMSO) and the adenosinergic drugs were dissolved in artificial cerebrospinal fluid (aCSF) with the aid of a minimal volume of DMSO (1% v/v final concentration in aCSF). Previous studies in our laboratory have shown no impairment of motor coordination with intracerebellar administration of up to 1 μl of 100% dimethylsulfoxide. The composition of the aCSF was (in mM): NaCl, 127.65; KCl, 2.55; $CaCl_2$, 0.05; $MgCl_2$, 0.94; $Na_2S_2O_5$, 0.05 (at pH 7.4). CP55,940, used for i.p. injection, was dispersed in a Tween-80 and 0.9% saline vehicle (1:19 v/v). CHA, used for i.p. injection, was

dissolved in 0.9% saline with the aid of a minimal volume of DMSO (0.2% v/v final concentration in saline).

2.4. Intracerebellar microinjections

Drugs were microinjected through PE-10 (Clay Adams) polyethylene tubing by a microinjection pump (Model 22, Harvard Apparatus, MA) fitted with 25-μl Hamilton syringes. The sterile stainless steel injection cannula (30 gauge; 0.31 mm diameter) was fitted to the PE-10 tubing so that the total length of exposed cannula was 11 mm. This allowed for protrusion of the injector cannula 1 mm. beyond the lower tip of the guide cannula. The volume of microinjection was 0.5 μl for CP55,940 which was determined in a separate study to be an optimal volume for a reproducible motor impairment response (unpublished observation). The other drugs were administered in a volume of 0.2 μl. The rate of microinjection was 0.5 μl/min for all microinjections. An air bubble separating drug solution and water in the tubing was monitored for continuous movement to indicate that blockage was not occurring and that the desired drug dose was administered.

2.5. Chronic i.p. treatment

Mice received 3 days of i.p. CP55,940 (0.1, 1 or 2 mg/kg) or CHA (0.25 or 1 mg/kg) in order to establish tolerance or cross-tolerance to the motor incoordination produced by an acute ICB microinjection of CP55,940. Injections were made daily at 16:00 h for once daily dosing (total of three injections in 3 days) and at 08:00 and 16:00 h for twice daily dosing (total of six injections in 3 days). Corresponding control groups received i.p. vehicle (0.1 ml/10 g body weight). Microinjections and subsequent rotorod evaluation occurred on day 4 approximately 16 h after the last i.p. injection of CP55,940, CHA or vehicle.

2.6. Motor incoordination evaluation

Mice were evaluated for motor coordination using a standard rotorod treadmill (Ugo Basill, Verese, Italy) set at a fixed speed of 24 rpm. As previously described [7,8], normal motor coordination was arbitrarily defined as the ability of a mouse to walk continuously on the rotorod, without falling off, for 180 s. All mice were screened prior to ICB microinjection to establish normal motor coordination and therefore acted as their own controls. The screening was performed the morning of the experiment typically 30 min prior to microinjections and subsequent rotorod evaluations. Any mouse unable to walk 180 s in three consecutive attempts during screening was considered to have abnormal coordination and was excluded from the experiment. Greater than 98% of the mice successfully screened for normal coordination on the morning of the experiments. Motor coordination experiments were performed between 08:00 and 11:00 h.

The rotorod evaluation times used were 15, 35 and 55 min starting from the moment of CP55,940 microinjections. After evaluation on the rotorod at each time point, mice were placed back into their original cages until the next evaluation time. Each treatment group consisted of 6–10 mice based on two rotorod experiments.

2.7. Tissue punch analysis of [^{3}H]CP55,940 dispersion

A solution (0.5 μl) containing [^{3}H]CP55,940 (0.1 μCi) and non-radioactive CP55,940 20 μg was microinjected into the same site of the cerebellum as the drugs used in the rotorod experiments. Mice (n=3) were killed by cervical dislocation 55 min later and decapitated. The brain was rapidly removed on a cold plate and then immediately frozen by immersion in a dry ice–ethanol solution. The brain was dried and rapidly mounted onto a freezing stage (Model #BFS-3TC; Physiotemp Instruments, Clifton, NJ), set at −12°C, and cut into 0.5-mm coronal sections with a sliding microtome (Model # 860; American Optical Co., Buffalo, NY). The sections were kept frozen (−20°C) on glass microscope slides.

Tissue punches of the cerebellar tissue in each coronal section were performed based on a modification of a previously described method [15,24]. Punches were made with thin-walled stainless steel tubes (21 gauge, 0.6 mm internal diameter; Small Parts, Miami, FL) beginning at the central site of microinjection in each 0.5 mm coronal section on a glass slide set on the freezing stage at −10°C. Punches were made 1 mm lateral, dorsal and ventral from the center of the central punch. A punch representing the deep cerebellar nuclei level was made 2 mm ventral from the central punch. A tissue punch was also taken from the brainstem of each section. The punched brain tissue was pushed out of the tube with a cold stainless steel wire (diameter 0.56 mm; Small Parts) into a scintillation vial containing 100 μl of 0.1 N NaOH. The tissue punches were incubated overnight (approximately 12 h) at 45°C in an Isotemp Oven (Fisher Scientific Co.). Five milliliters of Cytoscint liquid scintillation solution was then added. The vials were counted by a Beckman LS9000 liquid scintillation unit (Beckman Instruments, Fullerton, CA). Background counts, taken from the motor cortex, were subtracted from each punch made in the cerebellum and brainstem. Background counts did not exceed 18 dpm.

2.8. Histology

Mice were microinjected with green dye at the end of the experiments in order to confirm the accuracy of the drug microinjections. The mice were killed by cervical dislocation after light ether anesthesia. The guide cannula and brain were carefully removed and the site of microinjection was then verified by examining the location of

the dye in the cerebellar anterior lobe region. Only mice verified to have correct cannula placement were included in data analyses. The cannulation success rate was in excess of 95% in these studies.

Additionally, several mice from each treatment group were used for coronal sectioning of the cerebellum by microtome. The brains of mice were quickly removed and frozen in a dry ice–ethanol mixture and mounted for sectioning. The coronal sections were thaw-mounted onto glass slides and stained with cresyl violet. Examination under light microscope allowed for evaluation of any gross tissue damage caused by either the cannulation or drug microinjections. Only minimal variation in sites of microinjections and tissue damage due to cannula placement was observed between and within groups and treatments. This was consistent with previous cerebellum cannulations performed in our laboratory [9,10].

2.9. Statistical analyses

Motor incoordination data were analyzed by two-way ANOVA with factors of treatment and time and repeated measures on time. A Student's *t*-test was also used where appropriate. A Newman–Keuls post hoc test was performed whenever significance was found on treatment and/or time. A P value of less than 0.05 was taken as the level of significance in all statistical tests.

3. Results

3.1. Rotorod experiments

The doses of CP55,940 used in the present experiments (15 and 20 μg) were selected based on a previous dose–response analysis [13]. The 15-μg dose was established to be the median dose in production of motor incoordination. The 20-μg dose produced a more pronounced motor incoordination and was therefore deemed appropriate for use in the tolerance experiments with systemic CP55,940.

Fig. 1 shows the effects of ICB microinjection of CHA and DPCPX on CP55,940-induced motor incoordination. A significant treatment interaction occurred between groups receiving CHA or DPCPX+CP55,940 and vehicle+CP55,940 ($F_{3,29}=18.28$; $P<0.0001$). A time interaction was also apparent ($F_{2,29}=10.56$; $P<0.0001$). Post hoc analysis revealed that CHA, an A_1 receptor selective agonist, significantly accentuated the motor incoordination induced by CP55,940 at all three evaluation times ($P<0.01$) while DPCPX, an A_1 receptor-selective antagonist, significantly attenuated the motor incoordination at all three evaluation times ($P<0.01$). Microinjection of DPCPX also completely blocked the accentuation of CP55,940-induced motor incoordination by CHA. There was no significant recovery from motor incoordination even at 55 min post-microinjection in mice treated with

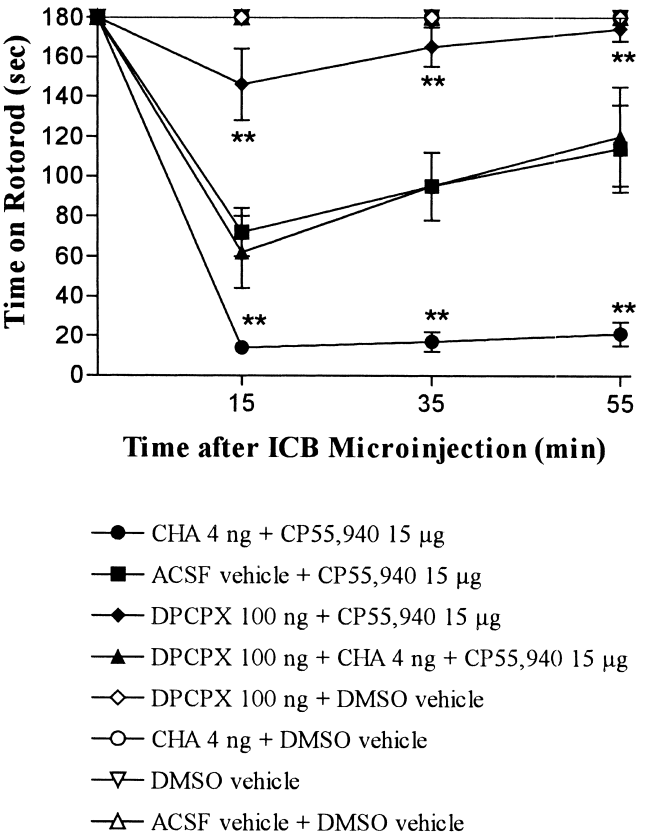

Fig. 1. Effect of A_1 receptor selective agonist CHA and/or A_1 receptor antagonist DPCPX on CP55,940-induced motor incoordination following intracerebellar microinjection. Each point represents the mean±S.E.M. of 6–9 mice. Significant accentuation by CHA occurred at all three evaluation times (**$P<0.01$, when compared to aCSF vehicle+CP55,940). Significant attenuation was noted with DPCPX at all three evaluation times (**$P<0.01$, when compared to vehicle+CP55,940).

CHA+CP55,940. This was in contrast to the vehicle+CP55,940 group which displayed significantly less motor incoordination at 55 min versus 15 min post-microinjection ($P<0.05$) thus signifying partial recovery or a trend toward recovery from peak motor incoordination (Fig. 1). The selection of doses for CHA and DPCPX was based on previously published work [8,9]. There was no change in normal motor coordination when ICB microinjection of CHA or DPCPX was followed by ICB microinjection of vehicle instead of the CP55,940 test dose (i.e. the animals were able to walk on the rotorod for 180 s).

Fig. 2 illustrates the effect of ICB microinjection of the adenosine transport inhibitor, dipyridamole, on CP55,940-induced motor incoordination. Dipyridamole had a significant treatment effect on CP55,940-induced motor incoordination ($F_{3,28}=10.28$; $P<0.0001$). Although the accentuation of motor incoordination by the 10-μg dose of dipyridamole was not statistically significant, the 25-μg dose accentuated the motor incoordination at all three evaluation times ($P<0.05$). In contrast, the highest dose, 50 μg, attenuated the motor incoordination by CP55,940 15 μg at the 35- and 55-min evaluation times ($P<0.01$).

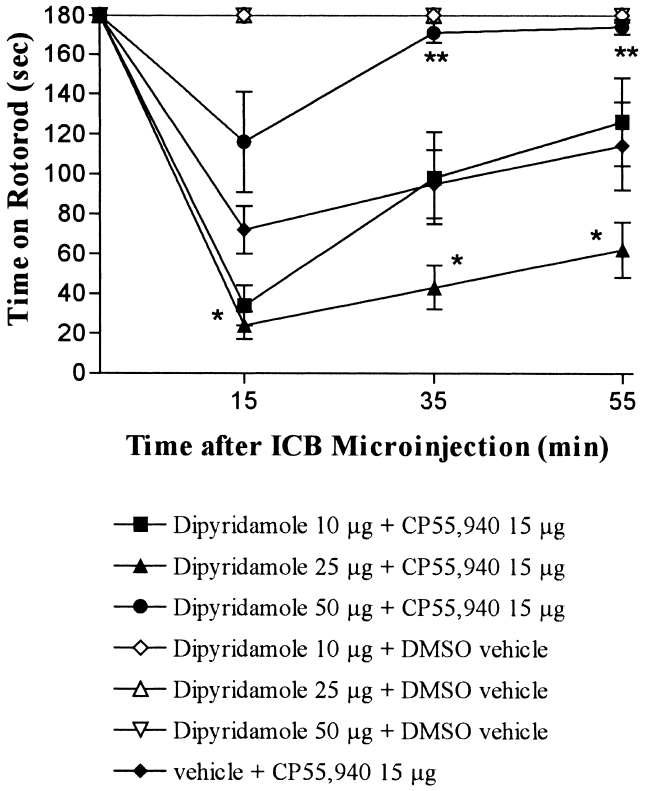

Fig. 2. Effect of various doses of dipyridamole on CP55,940-induced motor incoordination following intracerebellar microinjection. Each point represents the mean±S.E.M. of 6–9 mice. Significant accentuation was observed with the 25-μg dose at all three evaluation times (*$P<0.05$, when compared to vehicle+CP55,940) while significant attenuation was noted with the 50-μg dose at the 35- and 55-min evaluation times (**$P<0.01$, when compared to vehicle+CP55,940).

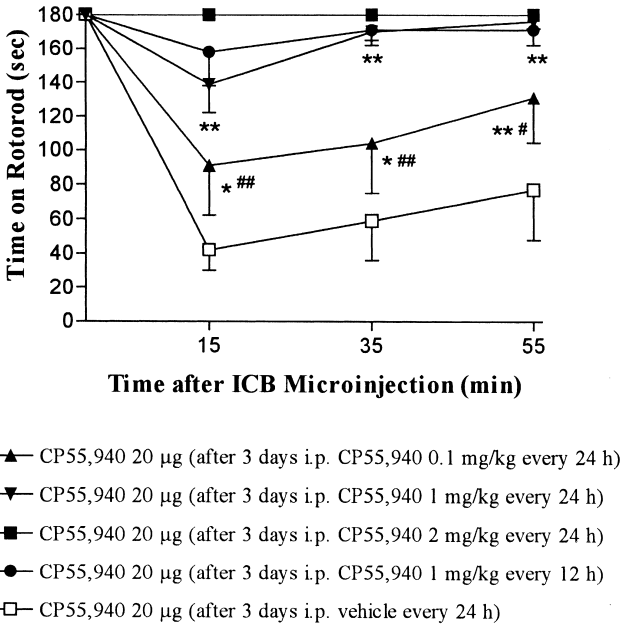

Fig. 3. Effect of 3-day, every 12 or 24 h, i.p. injection of CP55,940 on ICB CP55,940-induced motor incoordination. Each point represents the mean±S.E.M. of at least six mice. Significant tolerance was observed following 0.1, 1 or 2 mg/kg i.p. CP55,940 at all three evaluation times (*$P<0.05$, **$P<0.01$, when compared to the corresponding vehicle-treated group). The tolerance to ICB CP55,940 following the 3-day i.p. 0.1 mg/kg dosage was significantly lower in magnitude than the tolerance observed following the 3-day 1 mg/kg or 2 mg/kg dosage, indicating a dose dependence of the tolerance regimen ($^{\#}P<0.05$, $^{\#\#}P<0.01$, when compared to the 3-day 1 mg/kg or 2 mg/kg groups).

None of the three doses of dipyridamole produced any motor incoordination when administered prior to DMSO vehicle instead of CP55,940.

Fig. 3 shows the acute effects of ICB CP55,940 following chronic treatment for 3 days of i.p. CP55,940 at 0.1, 1 or 2 mg/kg. A significant tolerance occurred to the motor incoordinating effects of ICB CP55,940 20 μg following 3 days of once daily (every 24 h) i.p. CP55,940 at dosages of 0.1 and 1 mg/kg ($F_{3,23}=8.196$; $P=0.0007$). Post hoc analysis indicated the effect was significant at all three evaluation periods ($P<0.01$). A significant treatment×time interaction did not occur ($F_{6,208}=0.570$; $P=0.752$). The tolerance effect was dose-dependent as illustrated in Fig. 3. Once daily dosing of CP55,940 2 mg/kg induced complete tolerance to the effects of ICB CP55,940 20 μg at all evaluation times as indicated by mice remaining 180 s on the rotorod (Fig. 3) ($t=3.49–11.41$, $P<0.01$). There was also no change in the magnitude of motor incoordination produced by ICB CP55,940 (20 μg) following 3 days of i.p. vehicle when compared to those receiving ICB CP55,940 (20 μg) alone without prior vehicle treatment (data not shown).

Fig. 4 demonstrates a significant tolerance to the effects of ICB CHA 4 ng+CP55,940 15 μg following once daily dosing of i.p. CP55,940 1 mg/kg versus vehicle-treatment ($F_{2,18}=124.9$; $P<0.0001$). ICB CHA 4 ng+CP55,940 15 μg produced a small, but significantly greater motor incoordination effect than ICB vehicle+CP55,940 15 μg only at the 15-min evaluation time ($P<0.05$) in identically tolerized mice. In contrast, the motor incoordination was not significantly different at the 35- and 55-min evaluation times. This was in sharp contrast to the acute (Fig. 1) and chronic vehicle situation (Fig. 4) where CHA drastically accentuated the motor incoordination at all three evaluation times.

Fig. 5 demonstrates a significant treatment effect of 3 days i.p. CHA on ICB CP55,940 15 μg-induced motor incoordination. Administration of i.p. CHA, at dosages of 0.25 or 1 mg/kg every 24 h, induced significant cross-tolerance to the motor incoordinating effect of ICB CP55,940 15 μg ($F_{3,25}=35.69$; $P<0.0001$). Post hoc analysis on the 0.25 mg/kg dosage revealed significant cross-tolerance at the 15- and 35-min time periods ($P<0.01$). All mice treated with 1 mg/kg CHA for 3 days were able to walk 180 s at the 35- and 55-min evaluation periods following ICB CP55,940 15 μg. In addition, CHA 4 ng did not significantly accentuate the effect of ICB CP55,940 15 μg in mice treated with CHA 1 mg/kg i.p.

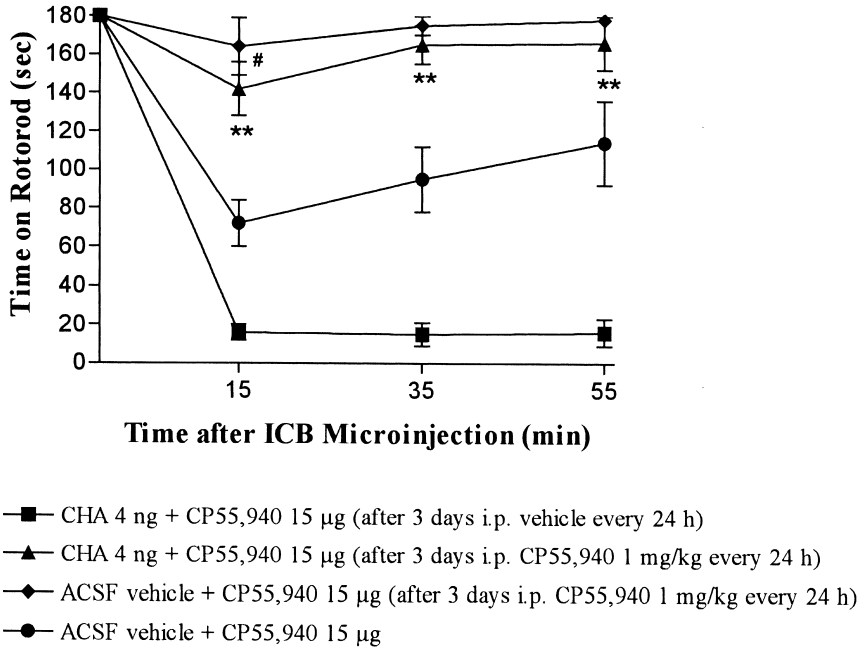

Fig. 4. Effect of 3-day, every 24 h, i.p. injection of CP55,940 1 mg/kg on motor incoordination produced by ICB CHA 4 ng+CP55,940 15 μg or ICB aCSF vehicle+CP55,940 15 μg. Each point represents the mean±S.E.M. of at least six mice. Significant tolerance was observed in the CP55,940-treated group at all three evaluation times (**$P<0.01$, when compared to the corresponding vehicle-treated group). The motor incoordination produced by ICB CHA+CP55,940, although minor, was slightly greater than ICB vehicle+CP55,940 at the 15-min evaluation time ($^{\#}P<0.05$).

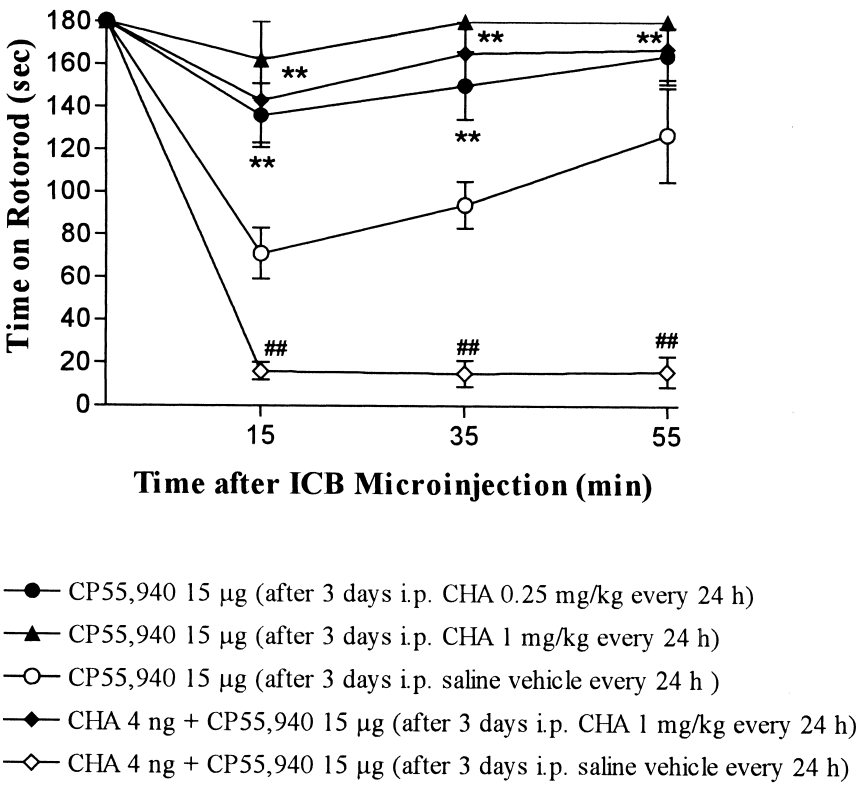

Fig. 5. Effect of 3-day, every 24 h, i.p. injection of A_1 receptor selective agonist, CHA 0.25 or 1 mg/kg, on ICB CP55,940-induced motor incoordination. Each point represents the mean±S.E.M. of at least six mice. Significant cross-tolerance was observed in the CHA-treated group at all three evaluation times (**$P<0.01$, when compared to the corresponding vehicle-treated group). ICB CHA 4 ng+ICB CP55,940 15 μg in vehicle-treated mice produced significantly greater motor incoordination than ICB CHA 4 ng+CP55,940 15 μg in CHA 1 mg/kg-treated mice ($^{\#\#}P<0.01$).

Table 1
Dispersion of [^{3}H]CP55,940 following ICB microinjection

	Dpm[a]				
Distance from Bregma (mm):	−5.4	−5.9	−6.4	−6.9	−7.4
Central site	18	1587	27656	708	14
1 mm dorsal	0	83	526	482	0
1 mm lateral (left)	1	2168	5272	214	11
1 mm lateral (right)	0	201	418	49	0
1 mm ventral	0	500	6780	154	3
Deep cerebellar nuclei	6	88	116	42	13
Brainstem	0	0	0	0	0

Representative dispersion pattern of CP55,940 ([^{3}H]CP55,940 0.1 μCi+ non-radioactive CP55,940 20 μg) in the cerebellum 55 min after intracerebellar (ICB) microinjection. The coronal section at −6.4 mm from bregma represents the central site of microinjection. The majority of [^{3}H]CP55,940 was found at the central site of microinjection. No significant counts above background were detected in the brainstem as evident in the table.

[a] Net dpm after subtracting the background counts (18 dpm) using motor cortex punched tissue.

for 3 days at the 15-min evaluation time (t=0.689, P= 0.508).

3.2. [^{3}H]CP55,940 dispersion in cerebellum

Table 1 represents a typical pattern of dispersion of ICB [^{3}H]CP55,940 at 55 min post-microinjection. [^{3}H]CP55,940 remained confined to a small area around the central region of the microinjection site in the cerebellum. Amounts of [^{3}H]CP55,940 were only slightly above background in coronal sections taken 1 mm anterior or posterior from the central site of microinjection. Very low net counts were found in punches corresponding to deep cerebellar nuclei (less than 150 dpm). No counts above background were found in the brainstem, a region outside of the cerebellum.

4. Discussion

The results of this investigation support the hypothesis of an adenosinergic modulation of CP55,940-induced motor incoordination. An acute interaction was noted following ICB microinjection of an A_1 receptor selective agonist (CHA) and/or antagonist (DPCPX) prior to ICB CP55,940. Furthermore, repeated systemic injection of CHA resulted in cross-tolerance to CP55,940.

CHA accentuated the motor incoordination produced by CP55,940 at all three evaluation times (see Fig. 1). It should be noted that the 4 ng CHA dose, administered alone, had no effect on motor coordination presumably because the effects on the receptor's signal transduction cascade were below threshold in producing motor incoordination in the absence of the cannabinoid agonist, CP55,940. When CHA was microinjected immediately prior to CP55,940, the effect on the signal transduction cascade became apparent by way of the enhanced motor incoordination observed upon rotorod evaluation. The accentuation likely occurred through the A_1 receptor, as opposed to A_{2a} or A_3 adenosine receptors, because microinjection of DPCPX completely blocked the accentuating effect of CHA (Fig. 1). DPCPX, an A_1 selective antagonist, has been reported to have a 740-fold higher affinity for the A_1 receptor over the A_{2a} receptor [5] and the rodent adenosine A_3 receptor subtype has been shown to be insensitive to the antagonist action of DPCPX [22]. Furthermore, the A_1 receptor is highly expressed in the molecular layer of the cerebellum whereas the A_{2a} is predominantly localized to the striatum and olfactory tubercles. The posterior cerebellum lobules may also contain some A_{2a} receptors however our microinjections targeted the anterior lobe region where adenosine A_{2a} receptors are not localized [31].

The mechanism of accentuation by CHA of CP55,940-induced motor incoordination was not investigated in the present study. An interaction at the level of the signal transduction pathway on the same synapses or at different synapses are both plausible explanations based on the fact that these receptors are located on several different neuron types in the cerebellum [23]. However, given the fact that the granule cells make up the majority of the neuronal population in the cerebellar molecular layer, it seems likely that transmission at granule cell parallel fiber synapses is an important target of action [26]. The A_1 receptor is known to be localized on the same parallel fiber terminals as the CB_1 receptors and utilizes the same signal transduction cascade as the CB_1 receptor ($G_{i/o}$ protein-adenylate cyclase-cAMP) [1,34].

We speculate that $G_{i/o}$ proteins and the cAMP system are involved in the behavioral interaction between CHA and cannabinoids. Previous research in our laboratory has clearly indicated that A_1 adenosinergic accentuation of ethanol-induced motor incoordination is dependent on $G_{i/o}$ proteins and inhibition of the cAMP pathway in the cerebellum [9]. It is then conceivable that ICB CHA+ CP55,940 resulted in greater inhibition of the adenylate cyclase-cAMP cascade than ICB CP55,940 alone. CP55,940 and CHA may each activate their own receptors and produce activation of their specific $G_{i/o}$ proteins coupled to their respective receptors. Both of these agonist drugs most likely interact at the post-receptor level at $G_{i/o}$ proteins and/or adenylate cyclase to influence neurotransmission in the cerebellum. An additive interaction between CB_1 and κ opioid receptors has already been described at the G-protein level with a less than additive interaction at the level of the adenylate cyclase enzyme [26]. It is plausible that this kind of interaction may occur between the CB_1 and A_1 receptor systems and be manifested behaviorally as a potentiation of CP55,940-induced motor incoordination. Furthermore, these $G_{i/o}$-coupled receptors are both thought to modulate glutamate release following activation by their respective endogenous ligands or ex-

ogenous agonists [4,29,32]. Inhibition of N-type calcium channels through a cAMP-independent mechanism and modulation of potassium current and membrane neurotransmitter release machinery through a cAMP-dependent pathway have both been cited as potential mechanisms of modulation of neurotransmitter release from synaptic terminals by cannabinoids [12,32] and adenosine A_1 agonists [4]. Recent electrophysiological studies have not implicated the cAMP system as being a dominant player in inhibition of glutamatergic transmission at the parallel fiber–Purkinje cell synapse [32].

The DPCPX attenuation of CP55,940-induced motor incoordination implies that the motor action of cannabinoids may be dependent, in part, on a tonic action of cerebellar adenosine acting through the A_1 receptor (see Fig. 1). It is also possible that the release of endogenous adenosine may be enhanced by cannabinoids which can then act through A_1 receptors and complement the signaling through the CB_1 receptors by inhibiting N-type Ca^{2+} channels and adenylate cyclase. Therefore, DPCPX may be acting by competitively antagonizing the effects of enhanced synaptic adenosine induced by cannabinoid agonists at the A_1 receptor. In theory, blockade of the A_1 receptors by DPCPX would result in less activation of $G_{i/o}$ proteins and therefore lessen the inhibition of the adenylate cyclase enzyme. It is at this post-receptor level where the interaction between the cannabinoid and adenosine systems likely occurred because there is no evidence indicating affinity of DPCPX for the CB_1 receptor. Additional study will be necessary to address the possibility of cannabinoid-induced increases in synaptic adenosine although factors affecting synaptic adenosine levels are thought to be numerous [4].

Further evidence of endogenous adenosine modulation was demonstrated in our laboratory by ICB microinjection of dipyridamole, an adenosine transport inhibitor. The 25-μg dose significantly enhanced motor incoordination at all evaluation times (see Fig. 2). Dipyridamole, by blocking the uptake of released adenosine, may have allowed enhanced activation of adenosine A_1 receptors due to accumulation of adenosine in the synapse. Enhanced activity at the A_1 receptor could then presumably allow for further inhibition of N-type Ca^{2+} channels and adenylate cyclase through increased stimulation of A_1 receptor-coupled $G_{i/o}$ proteins. It should be noted that 50 μg of dipyridamole, the highest dose administered, paradoxically attenuated the cannabinoid-induced motor incoordination at the 35- and 55-min evaluation times. This may have resulted from inhibition of phosphodiesterases [18] by the higher concentration of dipyridamole resulting in a counterbalancing effect on the cannabinoid-induced inhibition of cAMP accumulation. Therefore, although increased adenosine in the synapse due to dipyridamole acting through the A_1 receptor may have decreased cAMP production, we can speculate that the effect may have been overcome by concurrent inhibition of phosphodiesterases and subsequent accumulation of cAMP at the highest dose of dipyridamole. Increases in intracellular cAMP may act to enhance neurotransmission by increasing the probability of neurotransmitter release from the axon terminal [6]. This would be an effect opposite to that of cannabinoid-induced decreases in cAMP and subsequent inhibition of neurotransmission.

Tolerance to the behavioral effects of cannabinoids has been reported to develop quite rapidly and is often very marked in intensity [2]. This was clearly evident in our study of tolerance to the motor incoordinating effects of cannabinoids when we identified an optimal systemic administration regimen of CP55,940 that induced significant tolerance to the motor incoordinating effect of ICB CP55,940. The 0.1 and 1 mg/kg dosage of CP55,940 administered every 24 h for 3 days induced significant tolerance whereas the 2 mg/kg dosage resulted in complete tolerance as illustrated in Fig. 3. It is interesting to point out that the observed tolerance was dose-dependent. The higher dosages (1 and 2 mg/kg) resulted in significantly greater tolerance to the motor incoordinating effect of CP55,940 than the 0.1 mg/kg dosage. Several mechanisms of tolerance have been proposed including receptor uncoupling, receptor down-regulation and decreased expression of $G_{i/o}$ proteins [3,14,16].

We speculated that cross-tolerance would occur upon repeated exposure to systemic CP55,940 because of the acute adenosinergic system interaction with cannabinoids observed in the present investigation. Indeed, repeated exposure to i.p. CP55,940 resulted in only a small enhancement of residual ICB CP55,940-induced motor incoordination by ICB CHA at the 15-min evaluation time and no enhancement at 35 and 55 min post-microinjection (see Fig. 4). This observation suggested a potential cross-tolerance between cannabinoids and adenosine. Subsequently, further evidence was provided by the observation that 3-day treatment of animals with CHA resulted in marked tolerance to the motor incoordinating effect of ICB CP55,940, thereby confirming the phenomenon of cross-tolerance (Fig. 5). Also of note is the lack of CHA-accentuation of residual CP55,940-induced motor incoordination in mice treated with 3 days of i.p. CHA 1 mg/kg. Indirectly, the observed cross-tolerance between CP55,940 and CHA reinforces our proposed hypothesis based on acute data that the cerebellar A_1 system modulates CP55,940-induced motor incoordination. It was logical to see cross-tolerance because the acute effects of ICB CP55,940 appeared to be dependent, in part, on a functional adenosine A_1 receptor mechanism. Furthermore, cross-tolerance is not a new concept in cannabinoid pharmacology as cross-tolerance was observed in studies using κ opioids and cannabinoids [27].

Chronic treatment with an adenosine agonist, such as CHA, can result in desensitization of the adenosine A_1 receptor [11,25]. In the present investigation, repeated treatment with CHA most likely would similarly produce

A_1 receptor desensitization. The failure of ICB CP55,940 to produce motor incoordination in chronic CHA-treated animals therefore suggested heterologous desensitization of both the A_1 and CB_1 receptors. Due to the co-localization of the A_1 and CB_1 receptors on cerebellar neurons, there may be an interaction in these receptor's signal transduction pathways which can explain the behavioral interaction demonstrated using the rotorod test. It is possible that chronic administration of CHA may result in decreased expression of $G_{i/o}$ α-subunits in addition to uncoupling and down-regulation of A_1 receptors [28,34]. If this occurred, it is conceivable that it would decrease the action of CP55,940 through the CB_1 receptor due to a diminished pool of $G_{i/o}$ proteins available for coupling to the adenylate cyclase-cAMP cascade. Further analysis at the biochemical level is necessary to explain the behavioral interaction between cannabinoids and adenosine in the cerebellum.

It is important to point out that ICB CP55,940 remained confined locally to the site of microinjection in the cerebellum. Table 1 illustrates a representative dispersion pattern of [^{3}H]CP55,940 in the mouse cerebellum. No net counts were detected in the tissue punch corresponding to the brainstem. A punch in the motor cortex was around 18 dpm in each mouse evaluated and this was taken as background. The other microinjected drugs in this study were presumed to act locally at the site of microinjection based on previous work which demonstrated microinjection of [^{3}H]DPCPX and [^{3}H]CHA remains confined within 1 mm from the site of microinjection in rat striatum [24].

In summary, our data support that the cerebellar A_1 adenosinergic system modulates cannabinoid-induced motor incoordination because it is accentuated by an A_1 adenosine receptor agonist and attenuated by an A_1 receptor antagonist. The role of A_1 modulation is further supported by our observation of cross-tolerance between CHA, an A_1 receptor selective agonist and CP55,940, a cannabinoid-agonist. The involvement of cerebellar adenosine A_1 receptors in the motor incoordination by cannabinoid agonists may possibly result from interaction at some point in the cerebellar post-receptors' signal transduction pathways.

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